PREPARATION AND ANTIGENIC PROPERTIES OF DEHYDROEPI-ANDROSTERONE SULFATE-BOVINE SERUM ALBUMIN CONJUGATES HAVING A LINKAGE THROUGH THE POSITION IN RING D*

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The preparation and antigenic properties of two different types of dehydroepiandrosterone sulfate-bovine serum albumin (BSA) conjugates in which the hapten is linked to the carrier protein through the C-17 or C-15 position have been described. Antibodies raised against these antigens in rabbits possessed the high affinity ($K_a = 1.43 \times 10^9 - 2.20 \times 10^9 \text{ M}^{-1}$) to dehydroepiandrosterone sulfate. Antiserum elicited by immunization with the hapten-15 β -BSA conjugate was specific to dehydroepiandrosterone sulfate, exhibiting no significant cross-reactivities for other sulfated steroids and no cross-reactions for free steroids and their glucuronides with an exception of 16 α -hydroxydehydroepiandrosterone 3-sulfate (16.8%).

Keywords—dehydroepiandrosterone sulfate; direct radioimmunoassay; specific antiserum; dehydroepiandrosterone 3-sulfate 17-hydrazone; 15 β -(2-carboxyethylthio)-dehydroepiandrosterone 3-sulfate; hapten-BSA conjugate; association constant; cross-reaction

Dehydroepiandrosterone (3 β -hydroxy-5-androsten-17-one) sulfate is a principal conjugate of C₁₉ steroids in human peripheral blood. The conventional method for the determination of conjugated steroids involves prior deconjugation and subsequent analysis of liberated free steroids. Direct radioimmunoassay of steroid sulfates without prior hydrolysis or solvolysis is an attractive subject to be explored, but the preparation of antisera specific to steroid sulfates has not yet been developed. In the preceding papers of this series we reported the preparation of antisera specific to estrogen ring D glucuronides and their use for direct radioimmunoassay.¹⁻³⁾ The design of a hapten-carrier conjugate in the similar fashion appeared to be applicable to production of antibody specific to dehydroepiandrosterone sulfate. The present paper deals with the preparation of anti-dehydroepiandrosterone sulfate antisera with use of antigens in which the hapten is linked to

bovine serum albumin (BSA) through the position in ring D remote from the sulfate bond at C-3.

MATERIALS AND METHODS

Chemicals and Reagents—[7-³H]-Dehydroepiandrosterone sulfate ammonium salt (spec. act., 24 Ci/mmol) was supplied by New England Nuclear (Boston, Mass.) and the radiochemical purity was checked by thin-layer chromatography (TLC) prior to use. Dehydroepiandrosterone sulfate potassium salt and other conjugated steroids were synthesized in our laboratories by the known methods. All the free steroids were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo). BSA Fraction V and bovine serum gamma-globulin were purchased from Sigma Chemical Co. (St. Louis, Mo.), Sephadex G-25 and LH-20 from Pharmacia Fine Chemicals (Uppsala, Sweden), complete Freund's adjuvant and other

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general reagents from Nakarai Chemicals Ltd. (Kyoto), respectively. All solvents were distilled prior to use.

Synthesis of Haptens — All melting points were taken on a micro hot-stage apparatus and uncorrected. Optical rotations were measured with a JASCO Model DIP-4 automatic polarimeter. Infrared (IR) spectra were obtained with a JASCO Model IRA-1 spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL Model PS-100 spectrometer at 100MHz (^1H). Abbreviations used s=singlet and m=multiplet. Silica gel HF and silica gel (70–230 mesh) (E.Merck AG, Darmstadt) were used for preparative TLC and column chromatography, respectively.

i) Dehydroepiandrosterone 3-Sulfate 17-Hydrazone (II): Hydrazine hydrate (40 mg) was added to a solution of K salt of dehydroepiandrosterone sulfate (I) (100 mg) in MeOH (5 ml) and the solution was stirred at 40° overnight. The resulting precipitate was collected by filtration and recrystallized from MeOH to give K salt of II (80 mg) as colorless prisms, mp 251–254° (dec.). $[\alpha]_D^{20}$ –8.2° ($c=0.24$, MeOH). NMR (1% solution in CD_3OD) δ : 0.92 (3H, s, 18- CH_3), 1.08 (3H, s, 19- CH_3), 4.18 (1H, m, 3 α -H), 5.44 (1H, m, 6-H). *Anal.* Calcd for $\text{C}_{19}\text{H}_{29}\text{KN}_2\text{O}_4\text{S}\cdot 3/2\text{H}_2\text{O}$: C, 50.98; H, 7.20; N, 6.26; S, 7.16. Found: C, 50.91; H, 6.99; N, 6.10; S, 7.27.

ii) 15 β -(2-Carboxyethylthio)dehydroepiandrosterone 3-Sulfate (IV): Chlorosulfonic acid (0.4 ml) in pyridine (4 ml) was added to a solution of 15 β -(2-carboxyethylthio)dehydroepiandrosterone (III)⁴⁾ (100 mg) in pyridine (1 ml) under ice-cooling and the solution was stirred for 2 hr. The reaction mixture was neutralized with 1 N NaOH and passed through an Amberlite XAD-2 column. After thorough washing with water, the desired product was eluted with MeOH. The eluate was subjected to column chromatography on silica gel using $\text{CHCl}_3/\text{MeOH}$ (10:1–5:1) as a solvent. The eluate was converted into Na salt by passing the aqueous solution through a Dowex 50W-X8

(Na^+ form, 100–200 mesh) column followed by purification by gel chromatography on Sephadex LH-20 using MeOH as a solvent. The crude product was triturated with MeOH to give Na salt of IV (58 mg) as colorless amorphous substance, mp 200–202° (dec.). $[\alpha]_D^{25}$ –48.1° ($c=0.47$, MeOH). NMR (1% solution in CD_3OD) δ : 1.09 (3H, s, 18- CH_3), 1.12 (3H, s, 19- CH_3), 2.70 (4H, m, 15 β - $\text{S}(\text{CH}_2)_2$ -), 4.20 (1H, m, 3 α -H), 5.50 (1H, m, 6-H). *Anal.* Calcd for $\text{C}_{22}\text{H}_{30}\text{Na}_2\text{O}_7\text{S}_2$: C, 51.15; H, 5.85. Found: C, 51.22; H, 6.01. IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} : 1720 (C=O), 1210, 1070 ($-\text{SO}_3^-$).

Preparation of Hapten-BSA Conjugates —

i) From II by the Glutaraldehyde Method: To a solution of II (50 mg) and BSA (100 mg) in 0.2 M phosphate buffer (pH 6.8) was added 1% aqueous glutaraldehyde (2 ml) dropwise and stirred at 4° overnight. The resulting solution was dialyzed against running water at 4° overnight. After removal of the precipitate by centrifugation, the supernatant was passed through a Sephadex G-25 column. The fractions containing protein were collected and lyophilized to give a hapten-BSA conjugate (85 mg) as fluffy powder. The molar ratio of steroid to BSA in the conjugate was spectrophotometrically (at 404 nm) determined to be 37 using the color reaction with conc. H_2SO_4 .

ii) From IV by the Mixed Anhydride Method: To a solution of IV (32 mg) in dimethylformamide (2.9 ml)–dioxane (2.1 ml) were added tri-*n*-butylamine (0.016 ml) and isobutyl chlorocarbonate (0.009 ml) under ice-cooling and the solution was stirred for 1 hr. To this solution was added BSA (70 mg) in water (4.2 ml)–dioxane (2.1 ml), and the solution was adjusted to pH 8.0 with 1 N NaOH and stirred under ice-cooling for 5 hr. The resulting solution was dialyzed against running water at 4° for 2 days and then lyophilized to give a hapten-BSA conjugate (89 mg) as fluffy powder. Thirty-two mol of IV was incorporated into 1 mol of BSA, as determined by spectrophotometric analysis in the manner described in i).

Immunization of Rabbits — Three domestic strain male albino rabbits (body weight, 2.5–3.0

TABLE I. *Properties of Antibodies*

Hapten-BSA conjugate	Titer	Production time	Binding affinity (K_a)
[C-17] Conjugate	1:20000	5 months	$1.43 \times 10^9 \text{M}^{-1}$
[C-15] Conjugate	1:40000	3 months	$2.20 \times 10^9 \text{M}^{-1}$

kg) were used for immunization with each antigen. The antigen (2 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into rabbits subcutaneously at multiple sites over the scapula and in the thighs. This procedure was repeated at intervals of 2 weeks for further 2 months and then once a month. The rabbits were bled 10 days after the booster injection. The sera were separated by centrifugation at 3000 rev./min for 10 min and stored at -20° . The antisera were thawed and diluted with 0.05 M borate buffer (pH 8.0) containing 0.06% BSA and 0.05% bovine serum gamma-globulin.

Assay Procedure — A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 50, 100, 500, 1000 and 2000

pg of non-labeled dehydroepiandrosterone sulfate and $[7\text{-}^3\text{H}]$ -dehydroepiandrosterone sulfate (70 pg, 10000 dpm). The 1:20000 or 1:40000 dilution of antiserum (0.25 ml) was added and the mixture was incubated at room temperature for 1 hr. After addition of 50% $(\text{NH}_4)_2\text{SO}_4$ solution (0.25 ml), the resulting suspension was allowed to stand at room temperature for 15 min and then centrifuged at 3000 rev./min for 10 min. A two-tenth ml aliquot of each supernatant was transferred into a counting vial containing Bray's scintillator⁵⁾ (10 ml) and the radioactivity was counted on a Beckman Model LS 7000 liquid scintillation spectrometer.

RESULTS AND DISCUSSION

An initial effort was directed to the synthesis of antigen for obtaining anti-dehydroepi-

Chart 1

TABLE II. *Per Cent Cross-Reaction of Anti-Dehydroepiandrosterone Sulfate Antisera with Selected Steroids*

Steroid	% Cross-reaction (50%)	
	Antisera ^{a)}	
	A	B
Dehydroepiandrosterone sulfate	100	100
5-Androstene-3 β ,17 β -diol 3-sulfate	67.5	9.6
16 α -Hydroxydehydroepiandrosterone 3-sulfate	13.7	16.8
Androsterone sulfate	0.07	0.002
Estrone sulfate	0.29	0.25
Estradiol 17-sulfate	0.05	<0.001
Estriol 3-sulfate	0.10	<0.001
2-Hydroxyestradiol 3-sulfate	0.01	<0.001
Cholesterol sulfate	<0.01	<0.001
Testosterone glucuronide	<0.001	—
Androsterone glucuronide	<0.001	—
Estrone glucuronide	<0.001	<0.001
Estradiol 3-glucuronide	<0.001	<0.001
Estradiol 17-glucuronide	<0.001	<0.001
Estriol 3-glucuronide	<0.001	—
Estriol 16-glucuronide	<0.001	<0.001
Pregnanediol 3-glucuronide	<0.001	<0.001
Dehydroepiandrosterone	<0.001	0.003
16 α -Hydroxydehydroepiandrosterone	<0.001	<0.001
Androsterone	<0.001	<0.001
5-Androstene-3 β ,17 β -diol	<0.001	<0.001
4-Androstene-3,17-dione	<0.001	0.01
Testosterone	<0.001	<0.001
5 α -Dihydrotestosterone	<0.001	<0.001
Estrone	<0.001	<0.001
Estradiol	<0.001	<0.001
Estriol	<0.001	—
Progesterone	<0.01	<0.001
Cortisol	<0.001	<0.001

a) Preparation of hapten-BSA conjugates used for immunization: A) from dehydroepiandrosterone sulfate 17-hydrazone by the glutaraldehyde method; B) from 15 β -(2-carboxyethylthio)dehydroepiandrosterone sulfate by the mixed anhydride method.

androsterone sulfate antiserum. As a preliminary experiment we attempted to prepare a hapten-BSA conjugate in which dehydroepiandrosterone sulfate is linked to protein through its 17-oxo group, remote from the sulfate bond and also readily available for conjugation. Dehydroepiandrosterone sulfate (I) was transformed by treatment with hydrazine hydrate into the 17-

hydrazone (II) which in turn was coupled with BSA employing glutaraldehyde as a cross-linking agent⁶⁾ to provide the desired hapten-BSA conjugate. Spectrophotometric analysis of the colored solution produced with conc. sulfuric acid revealed that satisfactory number of steroid molecules were covalently bound to each BSA molecule in this antigen.

Three rabbits were immunized with the hapten-BSA conjugate emulsified with complete Freund's adjuvant in the usual manner. Five months after an initial injection of antigen, all the rabbits yielded antibodies exhibiting remarkably increased binding activity to dehydroepiandrosterone sulfate. A preliminary test indicated that there was no substantial difference in the affinity and specificity among antisera elicited in the three rabbits. Accordingly, antiserum possessing the highest titer was selected for detailed characterization.

Evaluation of the titer was performed by incubating various dilutions of antiserum with a constant amount of [7-³H]-dehydroepiandrosterone sulfate. The dilution of antiserum which was able to bind 50% of the labeled antigen was defined as a titer. The binding affinity was determined by the reaction of various concentrations of the labeled antigen with a constant volume of diluted antiserum. The association constant (K_a) was estimated to be $1.43 \times 10^9 \text{ M}^{-1}$ by a Scatchard plot.⁷⁾ The properties of antibodies are listed in Table I. The specificity of antiserum was assessed by testing the ability of the closely related compounds to compete for binding sites on the antibody. The percent cross-reactions determined by the method of Abraham⁸⁾ are collected in Table II. The antiserum elicited in the rabbit was capable of discriminating dehydroepiandrosterone sulfate from free steroids and their glucuronides. Almost all steroid sulfates exhibited less than 0.29% of the relative activity of dehydroepiandrosterone sulfate with two exceptions. It is well documented in the literatures that the structure remote from the attached position of a carrier protein is readily recognized in animals immunized with a hapten-protein conjugate. The present results imply that the use of an immunologic complex having the sulfate moiety intact is responsible for production of antibody specific to sulfated steroids. On the other hand blockage of the 17-oxo group for the preparation of hapten-BSA conjugate appears to be reflected to the generation of antiserum exhibiting the significant cross-reactivity with 5-androstene- β , β ,

17 β -diol 3-sulfate (67.5%).

Recently, Rao *et al.*⁹⁾ reported that highly specific anti-dehydroepiandrosterone antiserum is raised in rabbits against the conjugate obtained by coupling 15 β -(2-carboxyethylthio)dehydroepiandrosterone with BSA. Accordingly, the use of antigen in which the sulfated steroid hapten is linked to BSA through the 15 β position was undertaken. 15 β -(2-Carboxyethylthio)dehydroepiandrosterone, obtainable from dehydroepiandrosterone in several steps by the known method,⁴⁾ was treated with chlorosulfonic acid in pyridine to provide the 3-sulfate (IV) without disturbing the C-15 substituent. The hapten was covalently linked to BSA by the mixed anhydride method developed by Erlanger *et al.*,¹⁰⁾ yielding the desired dehydroepiandrosterone sulfate-15 β -BSA conjugate. The rabbits were immunized with this antigen in the manner described above. The antiserum elicited for three months exhibited the high affinity for dehydroepiandrosterone sulfate with the association constant of $2.20 \times 10^9 \text{ M}^{-1}$. A dose-response curve for dehydroepiandrosterone sulfate was constructed with 40000 dilution of antiserum. The results on cross-reactions of antiserum with selected steroids are listed in Table II. It is evident from the data that the specificity of antiserum for dehydroepiandrosterone sulfate was significantly improved, although minor cross-reactivities with 5-androstene- β , β , 17 β -diol 3-sulfate (9.6%) and 16 α -hydroxydehydroepiandrosterone 3-sulfate (16.8%) still remained unsolved.

It is sufficiently substantiated that the immunologic specificity is less dependent on the individual steroid than on the particular functional group occupied. The previous studies verified that highly specific antisera discriminating the structure around the conjugated position of estrogen ring D glucuronide are elicited by immunization with the hapten-BSA conjugate having a linkage through the position remote from the glucuronyl moiety.¹⁻³⁾ In the present work it has been demonstrated that the use of an immunologic complex having the linkage remote from the sulfate bond is responsible for produc

tion of antibody sufficiently specific to sulfated steroid.

The design of a hapten-carrier conjugate in the similar fashion may be further applicable to the preparation of specific antisera to other conjugated steroids. Development of a simplified and reliable direct radioimmunoassay system without prior deconjugation for dehydroepiandrosterone sulfate in biological fluids employing specific antiserum will be the subject of a future communication.

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